

Exhibit 5

## Research Articles

# Combinatorial Cassette Mutagenesis as a Probe of the Informational Content of Protein Sequences

JOHN F. REIDHAAR-OLSON AND ROBERT T. SAUER

NOTICE: This material may be protected by copyright law (Title 17 U.S. Code)

A method of combinatorial cassette mutagenesis was designed to readily determine the informational content of individual residues in protein sequences. The technique consists of simultaneously randomizing two or three positions by oligonucleotide cassette mutagenesis, selecting for functional protein, and then sequencing to determine the spectrum of allowable substitutions at each position. Repeated application of this method to the dimer interface of the DNA-binding domain of  $\lambda$  repressor reveals that the number and type of substitutions allowed at each position are extremely variable. At some positions only one or two residues are functionally acceptable; at other positions a wide range of residues and residue types are tolerated. The number of substitutions allowed at each position roughly correlates with the solvent accessibility of the wild-type side chain.

IT HAS BEEN MORE THAN 20 YEARS SINCE ANFINSEN AND HIS colleagues showed that the sequence of a protein contains all of the information necessary to specify the three-dimensional structure (1). However, the general problem of predicting protein structure from sequence remains unsolved. Part of the difficulty may stem from the complexity of protein structures. Although some 200 protein structures are known, no rules have emerged that allow structure to be related to sequence in any simple fashion (2). The problem is further complicated by the nonuniformity of the structural information encoded in protein sequences. Some residue positions are important, and changes at these positions can tip the balance between folding and unfolding (3-7). Other residues are relatively unimportant in a structural sense and a wide range of substitutions or modifications can be tolerated at these positions (3, 7-9).

If only a fraction of the residues in a protein sequence contribute significantly to the stability of the folded structure, then it becomes important to be able to identify these residues. We now describe the results of genetic studies that allow the importance of individual residues in protein sequences to be rapidly determined. Specifically, we determine the spectrum of functionally acceptable substitutions at residue positions near the dimer interface of the  $\text{NH}_2$ -terminal domain of phage lambda ( $\lambda$ ) repressor (10). The  $\text{NH}_2$ -terminal domain binds to operator DNA as a dimer, with dimerization

mediated by hydrophobic packing of  $\alpha$  helix 5 of one monomer against  $\alpha$  helix 5' of the other monomer (11) (Fig. 1, A and B). Without helix 5 there are no contacts between the subunits (Fig. 1C). By applying combinatorial cassette mutagenesis to the helix 5 region, we find that the number and spectrum of allowable substitutions within helix 5 are extremely variable from residue to residue. In most cases, this variability can be rationalized in terms of the fractional solvent accessibility of the wild-type side chain.

**General strategy.** For our studies, we used a plasmid-borne gene that encodes a functional, operator-binding fragment (residues 1-102) of  $\lambda$  repressor (12). The binding of the 1-102 fragment to operator DNA depends on dimerization which, in turn, depends on the helix 5-helix 5' packing interactions (11, 13). Thus, if a 1-102 protein retains normal operator-binding properties, we can infer that it is able to dimerize normally.

Mutagenesis of the helix 5 region was performed by a combinatorial cassette procedure. One example of this method, in which codons 85 and 88 are mutagenized, is illustrated in Fig. 2. On the top strand, the mutagenized codons are synthesized with equal mixtures of all four bases in the first two codon positions and an equal mixture of G and C in the third position. The resulting population of base combinations will include codons for each of the 20 naturally occurring amino acids at each of the mutagenized residue positions. On the bottom strand, inosine is inserted at each randomized position because it is able to pair with each of the four conventional bases (14). The two strands are then annealed and the mutagenic cassette is ligated into a purified plasmid backbone.

To identify plasmids encoding functional protein, we selected transformants for plasmid-encoded resistance to ampicillin and for resistance to killing by  $dI^-$  derivatives of phage  $\lambda$ . The latter selection requires that the cell express 1-102 protein that is active in operator binding (15). For each mutagenesis experiment, many independent transformants were chosen, single-stranded plasmid DNA was purified, and the relevant region of the 1-102 gene was sequenced. The resulting set of sequences provides a list of functionally acceptable helix 5 residues.

**Substitutions in the helix 5 region.** In separate experiments with different mutagenic cassettes, the codons for helix 5 residues 85 and 88; 86 and 89; 90 and 91; 84, 87, and 88; and 84, 87, and 91 were mutagenized, and genes encoding active 1-102 proteins were selected. In some cases, the survival frequency was low. For example, only 17 of 60,000 transformants passed the selection after randomization of codons 84, 87, and 88. In this case, each active candidate was sequenced. By contrast, 1,200 of 50,000 transformants passed the selection in the mutagenesis of positions 86 and 89 (16). In this case, we picked 50 candidates for sequence analysis. Overall, 150 active genes were sequenced (Table 1). In addition, we sequenced

The authors are in the Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.



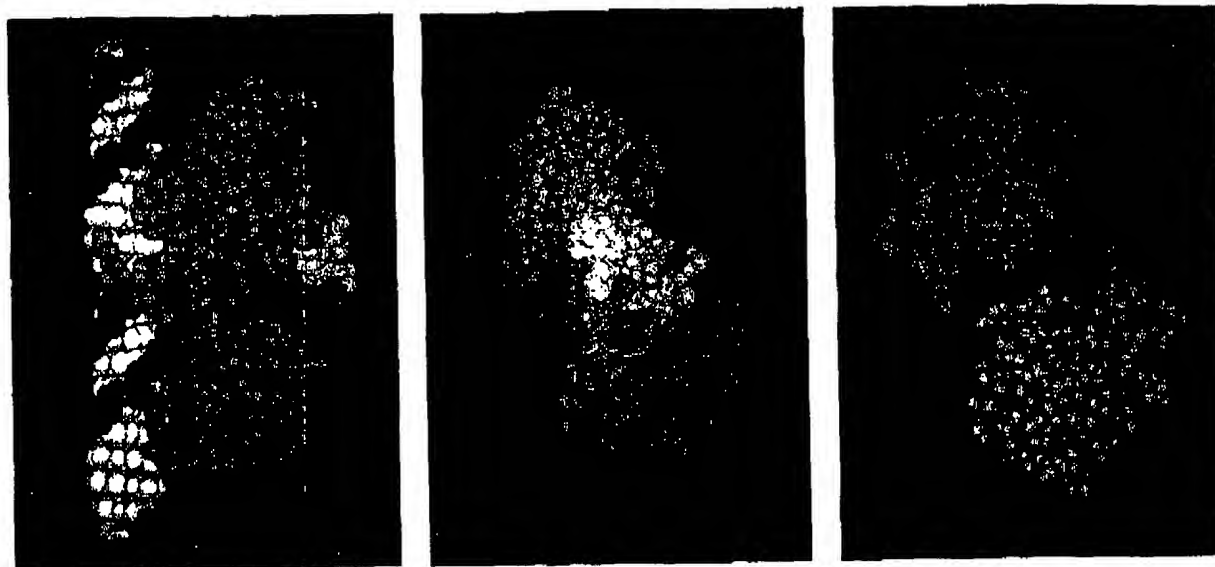
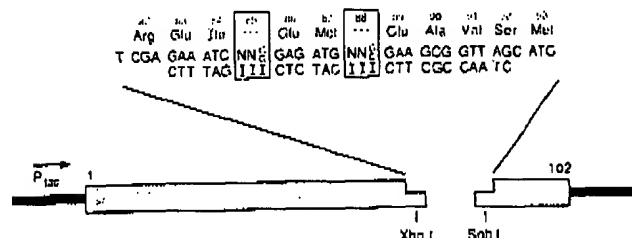


Fig. 1. Three views of the DNA-binding domain of  $\lambda$  repressor, showing the role of helix 5 in dimerization. (A) Proposed complex of repressor dimer with operator DNA (11). Helix 5 of each monomer is colored more lightly than the globular portion of that monomer. (B) Free repressor dimer,

rotated 90° from the view in (A), to show the "back side" of the molecule. (C) Dimer with helix 5 of each monomer removed. This view illustrates the role helix 5 plays in mediating dimerization (26).

Fig. 2. Schematic diagram showing the combinatorial cassette mutagenesis procedure. At positions indicated as N, an equal mixture of A, G, C, and T was used, during oligonucleotide synthesis. At positions indicated as I, inosine was used. After synthesis, the oligonucleotides were phosphorylated, annealed, and ligated into the Xho I-Sph I backbone of plasmid pJO103. Plasmid pJO103 is an M13 origin plasmid with the 1-102 gene under control of a *lac* promoter; the region of the 1-102 gene encoding residues 82-93 (the small Xho I-Sph I fragment) is replaced by an unrelated 1.9-kb Xho I-Sph I "stuffer" fragment. Ligated DNA was transformed into *Escherichia coli* strain X90 F' *lacI*<sup>q</sup> cells (27), and ampicillin-resistant colonies were selected in the presence or absence of phage  $\lambda$ KH54. Candidates that survived the selection were cross-streaked against a series of virulent derivatives of phage  $\lambda$  to confirm their immunity properties [strains and methods are described in (21)]. Single-stranded plasmid DNA was purified from an M13RV1 transducing lysate as described (28), and DNA sequences were determined by the dideoxy method (29).



the wild-type dimer, the side chains of Tyr<sup>85</sup>, Glu<sup>86</sup>, and Glu<sup>89</sup> are relatively solvent accessible.

Several amino acids are significantly underrepresented among the active sequences. For example, Pro is never found. This cannot be an artifact of our mutagenesis procedure because Pro is frequently observed among the unselected mutant sequences (Table 1). We conclude that Pro is not found among the functional sequences because it is selected against; its presence would presumably disrupt the  $\alpha$ -helical structure and thereby the helix-helix packing at the dimer interface.

His, Asn, and Lys are also underrepresented among the functional helix 5 sequences. These residues are presumably not acceptable at positions 84 and 87, where the informational content is extremely high, and may not be acceptable at positions 88 and 91, where the functional substitutions are generally hydrophobic in character. The acceptability of these residues at positions such as 85 and 86 is difficult to assess from our experiments because the codons for these residues are present at reasonably low frequencies even among the unselected sequences. In these cases, we probably have not sequenced a large enough number of candidates to be confident that all acceptable substitutions have been identified. In fact, data from reversion studies (21) and suppressed amber studies (22) show that His<sup>85</sup> and Lys<sup>86</sup> are acceptable substitutions in the context of the intact  $\lambda$  repressor molecule.

**Informational content and protein structure.** We have com-

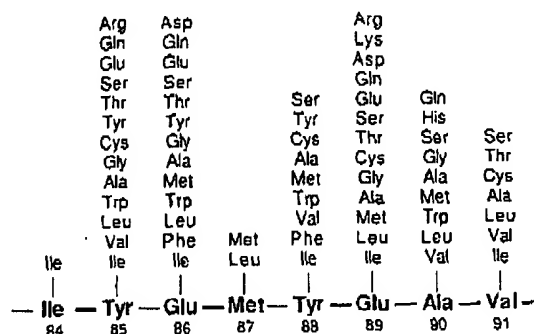


Fig. 3. Functionally acceptable residues in the helix 5 region. The amino acids are listed from top to bottom in order of increasing hydrophobicity according to the scale of Eisenberg *et al.* (30).

bined an efficient combinatorial mutagenesis procedure and a functional selection to probe the informational content of the eight residues that form the major part of the dimerization interface of the NH<sub>2</sub>-terminal, operator-binding domain of  $\lambda$  repressor. At two of these eight residue positions, the functionally acceptable choices are highly restricted. For example, we analyzed 17 functional genes in which codon 84 had been randomized and recovered the wild-type residue, Ile, in every case. This is clearly a position of high

Fig. 4. Residues high in informational content. The two isolated helix 5 regions of the protein are shown in green and blue. Ile<sup>84</sup> and Met<sup>87</sup> from the green helix are shown in yellow; Ile<sup>84</sup> and Met<sup>87</sup> from the blue helix are shown in red.

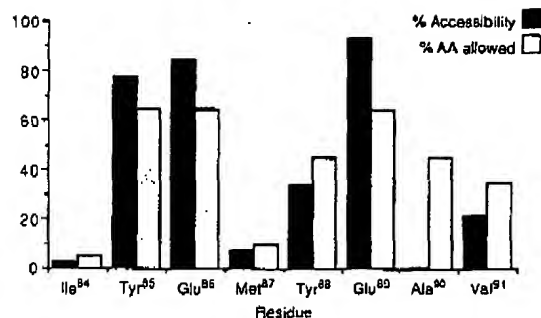
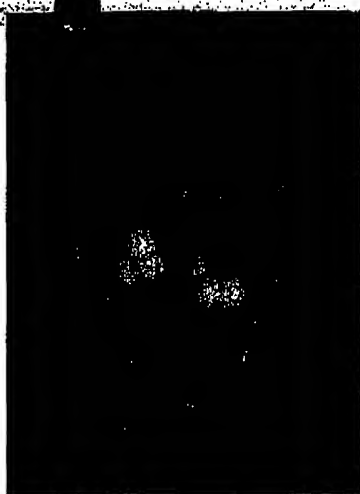


Fig. 5. Correlation between the solvent accessibility and the number of functionally acceptable substitutions. Hatched bars indicate the percentage of the 20 naturally occurring amino acids that are functionally acceptable at a residue position. Black bars indicate the fractional solvent accessibility of the wild-type side chain in the dimer. Solvent accessibilities for the NH<sub>2</sub>-terminal domain dimer (11) were computed using a 1.4 Å probe by the method of Lee and Richards (17). Fractional accessibilities were obtained by dividing by the appropriate side chain accessibilities calculated for the monomer. The fractional accessibilities change only slightly if the side chain accessibilities in the reference tripeptide Ala-X-Ala (17) are used instead as the reference state.

informational content. The informational content is also high at position 87, where Met and Leu are the only acceptable residues. By contrast, the remaining positions have moderate to low informational contents. For example, among 38 functional genes in which codon 85 had been randomized, the wild-type residue was recovered only once, and 12 other residues, differing in size and chemical properties, were recovered in the remaining cases. This is clearly a position of low informational content. It is striking that most of the structural determinants of dimerization in this eight-residue segment reside in two residues only. The remaining positions are surprisingly tolerant of a wide range of substitutions. If this high level of tolerance is generally true of protein sequences, then the problem of understanding and predicting structure may rest largely on the ability to identify those few residues that are crucial.

The positional variability of the informational content in helix 5 can, in general, be rationalized in terms of the solvent accessibility of the wild-type residues in the crystal structure (11). There is a rough correlation between the number of acceptable substitutions and the fractional extent to which the wild-type side chain is solvent accessible (Fig. 5). At exposed surface positions such as 85, 86, and 89, we find that many different residues and residue types can be functionally accommodated. By contrast, at positions such as 84 and

87, where the wild-type side chain is almost completely buried, we find that the functionally acceptable residue choices are extremely restricted. There is one apparent exception to the simple rule that buried residues are high in informational content. Ala<sup>90</sup> is inaccessible to solvent in the crystal structure, and yet we find that many substitutions are allowed at this position. However, the inaccessibility of the Ala<sup>90</sup> side chain to solvent is not due to close packing at the dimer interface, but rather to an interaction with a nearby surface side chain. This side chain can presumably move to allow larger side chains to be accommodated at position 90. Examples of this type demonstrate the need to distinguish between two types of buried side chains: those that can become exposed by relatively minor rearrangement of other side chains, and those that are tightly packed in the hydrophobic core.

There is no reason to assume that there should always be a strict correlation between the solvent accessibility of a residue and the structural informational content of that position. For one thing, the chemical properties of the 20 amino acids are not related in any simple linear fashion. Moreover, the structural importance of some residues in proteins almost certainly stems from interactions other than simple hydrophobic packing. Nevertheless, the closely packed nature of protein interiors (23) provides a simple molecular explanation for the structural importance of buried residues, and destabilizing mutations are commonly found to affect hydrophobic core residues (3-7). By contrast, missense mutations or chemical modifications that affect surface residues are often found to have little or no influence on protein stability (3, 7, 8). Thus, it is reasonable that solvent accessibility should be an extremely important determinant of the informational content of a residue position.

Our overall strategy for rapidly probing informational content should be broadly applicable to a wide range of protein structure-function problems in systems where genetic selections or screens can be devised. The method consists of three basic elements: (i) the use of cassette mutagenesis to introduce extremely high levels of targeted random mutagenesis; (ii) the use of a functional selection to identify genes encoding active proteins; and (iii) the use of rapid DNA sequencing methods to determine the spectrum of functionally acceptable residues in a relatively large number of candidates. Our method of combinatorial cassette mutagenesis (Fig. 2) allows several residue positions to be mutagenized at the same time and, in principle, generates a mutant population in which each of the 20 amino acids is represented at each mutagenized position (24). When two or three codons are mutagenized at the same time, the entire analysis is able to proceed more rapidly. Moreover, at this level of mutagenesis most two-residue and three-residue combinations should be present in the mutagenized population and should be recovered if they result in a functional protein. In our study of the packing of the 84 and 87 side chains, we recovered only two (Ile<sup>84</sup> with Met<sup>87</sup> and Ile<sup>84</sup> with Leu<sup>87</sup>) of the 400 possible residue combinations. Thus, because both positions were mutagenized in the same experiment, we are able to conclude that there are not significantly different ways of packing the dimer interface.

In principle, data like that shown in Fig. 3 could be generated for an entire protein sequence, and additional experiments could be devised to determine whether the positions of high informational content were important for structure or function. For proteins of unknown structure, such data might be quite useful for structural predictions. First, current predictive algorithms could be applied to the family of related sequences generated by our method, as each of these sequences is able to form the same basic structure. Second, because of their fundamental repeats,  $\alpha$ -helical and  $\beta$ -strand regions might be recognized by characteristic patterns of high and low informational content. Third, the positions of highest structural informational content should include the residues involved in

formation of the hydrophobic core of the protein. This information might prove useful in combination with the tertiary template ideas recently proposed (25).

## REFERENCES AND NOTES

1. C. J. Epstein, R. F. Goldberger, C. B. Anfinsen, *Cold Spring Harbor Symp. Quant. Biol.* 28, 439 (1963); C. B. Anfinsen, *Science* 181, 223 (1973).
2. T. E. Creighton, *Proteins: Structures and Molecular Properties* (Freeman, New York, 1983), chap. 6.
3. M. H. Hecht, H. C. M. Nelson, R. T. Sauer, *Proc. Natl. Acad. Sci. U.S.A.* 80, 2676 (1983); M. H. Hecht, J. M. Sturtevant, R. T. Sauer, *ibid.* 81, 5685 (1984); M. H. Hecht, K. M. Hehir, H. C. M. Nelson, J. M. Sturtevant, R. T. Sauer, *J. Cell. Biochem.* 29, 217 (1985).
4. D. Shorde and B. Lin, *Genetics* 110, 539 (1985); D. Shorde and A. K. Meeker, *Protein* 1, 81 (1986).
5. A. Pakula, V. Young, R. T. Sauer, *Proc. Natl. Acad. Sci. U.S.A.* 83, 8829 (1986).
6. T. Alber, S. Dao-pin, J. A. Nye, D. C. Muchmore, B. W. Matthews, *Biochemistry* 26, 3754 (1987).
7. G. Fermi and M. F. Perutz, *Haemoglobin and Myoglobin* (Clarendon, Oxford, 1981).
8. M. Hollacker and T. E. Creighton, *Biochem. Biophys. Acta* 701, 395 (1982).
9. J. H. Miller, in *The Operon*, J. H. Miller and W. S. Reznikoff, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1978), pp. 31-88.
10.  $\lambda$  repressor consists of an NH<sub>2</sub>-terminal and COOH-terminal domain; each domain contains an independent set of dimerization contacts [C. O. Pabo, R. T. Sauer, J. M. Sturtevant, M. Pashine, *Proc. Natl. Acad. Sci. U.S.A.* 76, 1608 (1979); C. O. Pabo, thesis, Harvard University (1980)].
11. C. O. Pabo and M. Lewis, *Nature* 298, 443 (1982).
12. The NH<sub>2</sub>-terminal domain of  $\lambda$  repressor is minimally comprised of residues 1-92. This is the fragment for which the crystal structure is known. In our studies we use a slightly larger NH<sub>2</sub>-terminal fragment consisting of repressor residues 1-102. Nuclear magnetic resonance studies show that the 1-92 and 1-102 fragments have the same basic structure and similar dimerization properties (13). The operator binding properties of the 1-92 and 1-102 fragments are also extremely similar (20).
13. M. A. Weiss, C. O. Pabo, M. Karplus, R. T. Sauer, *Biochemistry* 26, 897 (1987); M. A. Weiss, M. Karplus, R. T. Sauer, *ibid.*, p. 890.
14. F. H. Martin, M. M. Castro, F. Aboul-ela, I. Tinoco, *Nucleic Acids Res.* 13, 8927 (1985).
15. In control experiments, we found that cells containing at least 5 to 10 percent of the wild-type activity could survive the phage selection. This figure was determined by Western analysis of lysates of cells containing the wild-type 1-102 gene under control of the inducible *lac* promoter. When expression of 1-102 was induced to a level sufficient to make cells resistant to phage  $\lambda$ KH54, the intracellular level of 1-102 was 5 to 10 percent of that produced from the *lac* promoter.
16. The survival frequency can be somewhat misleading, as some cells containing functional genes do not survive the selection. For example, by screening the unselected candidates in the mutagenesis of positions 86 and 89, we found that approximately 20 percent of the cells contained active protein. By contrast, only 2.4 percent of the cells survived the selection in this experiment.
17. B. Lee and F. M. Richards, *J. Mol. Biol.* 55, 379 (1971); in our studies, accessible surface areas were calculated with the use of the ACCESS program written by T. Richmond.
18. F. S. Gimble and R. T. Sauer, *J. Mol. Biol.*, in press.
19. S. Jordan and C. Pabo, personal communication.
20. R. T. Sauer et al., *Biochemistry* 25, 5992 (1986); C. O. Pabo and E. G. Suchanek, *ibid.* 25, 5987 (1986).
21. M. H. Hecht and R. T. Sauer, *J. Mol. Biol.* 186, 53 (1985).
22. H. C. M. Nelson, M. H. Hecht, R. T. Sauer, *Cold Spring Harbor Symp. Quant. Biol.* 47, 44 (1983).
23. F. M. Richards, *J. Mol. Biol.* 82, 1 (1974); C. Chodha, *Nature* 254, 304 (1975).
24. There are several methods, in addition to our own, that could be used for combinatorial cassette mutagenesis: A. R. Oliphant, A. L. Nussbaum, K. Struhl, *Gene* 44, 177 (1986); K. M. Derbyshire, J. J. Salvo, N. D. F. Grindley, *ibid.* 46, 145 (1986). The use of inosines, in our method, is convenient and efficient, but does lead to some bias in the frequency at which each base is recovered. For example, among the unselected candidates in the mutagenesis of positions 84, 87, and 88, the frequencies at which the four bases were recovered at the mutagenized positions were: 23 percent A, 35 percent C, 20 percent G, and 23 percent T. The methods cited above avoid a pairing bias by performing enzymatic second-strand synthesis.
25. J. W. Ponder and F. M. Richards, *J. Mol. Biol.* 193, 775 (1987).
26. These graphics were produced with the Promodober molecular graphics program (New England BioGraphics). Coordinates were provided by C. O. Pabo.
27. D. Hanahan, *J. Mol. Biol.* 166, 557 (1983).
28. A. K. Vershon, K. Blackmer, R. T. Sauer, in *Protein Engineering: Applications in Science, Medicine, and Industry*, M. Inouye and R. Sarma, Eds. (Academic Press, Orlando, FL, 1986), pp. 243-256.
29. F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463 (1977).
30. D. Eisenberg, R. M. Weiss, T. C. Terwilliger, W. Wilcox, *Faraday Symp. Chem. Soc.* 17, 109 (1982).
31. We thank R. Breyer for providing plasmids and antibodies required for the work, D. Vershon for suggesting the use of inosines for the combinatorial mutagenesis method, and J. Bowie for pointing out the potential uses of the method in structural prediction. We also thank C. Pabo and S. Jordan for providing the coordinates of the NH<sub>2</sub>-terminal domain and its complex with operator DNA, and thank G. Quigley for help with the accessibility calculations. This work was supported by NIH grant AI-15706 and by a predoctoral grant (to J.R.-Q.) from the National Science Foundation.

3 March 1988; accepted 18 May 1988

